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Mediated electrochemical oxidation of glucose via poly(methylene green) grafted on the carbon surface catalyzed by flavin adenine dinucleotide-dependent glucose dehydrogenase

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ABSTRACT

Electrochemically polymerized phenothiazines (thionine, methylene green, methylene blue, and toluidine blue) on carbon electrodes were investigated as electron transfer mediators of glucose oxidation by flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) for biosensor and biofuel cell applications. Among the tested polyphenothiazines grafted on a glassy carbon electrode, clear redox-mediating activity was observed for poly(methylene green), and the catalytic oxidation current depended on the concentrations of glucose and enzymes and the amount of polymer deposited on the electrode surface. The poly(methylene green)-grafted porous carbon electrodes showed 3 mA cm^{-2} of glucose oxidation current catalyzed by FAD-GDH.

1. Introduction

The extensively investigated electrocatalytic oxidation of glucose is widely used in glucose biosensors and biofuel cells (BFCs). This process can be divided into three main types: enzymatic, microbial, and abiotic. Enzymatic electrocatalysis offers several advantages, such as selectivity, activity at near-room temperature/neutral pH, and high catalytic turnover [1,2]. The most commonly used enzyme for glucose oxidation is glucose oxidase (GOx), which is a stable enzyme with high selectivity. However, in the presence of oxygen and glucose, GOx catalyzes the oxidation of glucose and the reduction of oxygen to hydrogen peroxide, which can affect the sensitivity of mediator-assisted biosensors, induce a loss of coulombic efficiency in BFCs, and damage bioelectrodes. For these reasons, flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase (FAD-GDH) has attracted considerable attention as an electrocatalyst for glucose oxidation because it does not utilize oxygen as an electron acceptor [1,3–5]. Moreover, FAD-GDH exhibits high glucose selectivity and is capable of fast electron transfer to artificial electron acceptors [6]. In addition, unlike diffusional nicotinamide dinucleotide (NAD)-type GDH, FAD is deeply buried in the protein shell and strongly attached to it. Our previous study demonstrated that the rate constant of the bimolecular reaction between FAD-GDH and diffusional artificial organic electron acceptors, including quinones and phenothiazines, exceeds that of the reaction between GOx and the above acceptor by three orders of magnitude [6].

However, the use of bioelectrocatalysis by FAD-GDH for continuous-monitoring biosensors or for one-compartment-type or refuelable BFCs requires the co-immobilization of FAD-GDH and its redox mediator on the electrode surface [4,7,8]. Polymer-tethered and enzyme surface-grafted quinones have been recently reported as redox mediators for the bioelectrocatalytic oxidation of glucose by FAD-GDH [9,10]. However, such mediators require complex synthesis procedures, which makes the use of phenothiazine derivatives such as methylene blue and methylene green very attractive. Moreover, the use of these organic redox mediators offers the advantages of disposability, sustainability, and low cost. Therefore, these mediators are more desirable because they are inexpensive and stable molecules are easily polymerized on the electrode surface upon application of oxidative potentials. Although electrochemically polymerized phenothiazines are known to catalyze the oxidation of the diffusional redox cofactor NADH (NADPH) [11–17], their mediating function toward non-diffusional cofactors, such as FAD, has not yet been reported.

Herein, we electrochemically deposited several poly(phenothiazine) redox polymers on a glassy carbon (GC) electrode surface using thionine (TH), methylene green (MG), methylene blue (MB), and toluidine blue (TB) as starting monomer materials. We investigated the capability of these redox polymers in mediating electron transfer between FAD-GDH and electrodes. High-surface-area MgO-templated porous carbon [18–21] was also tested as a scaffold to increase the performance of the FAD-GDH-based poly(phenothiazine)-modified electrode for glucose

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oxidation.

2. Materials and methods

2.1. Materials

FAD-GDH isolated from *Aspergillus terreus* was donated by Ikeda Tohka Industries Co., Ltd. (Fukuyama, Japan) [3], and its concentrations were determined spectrophotometrically using the molar extinction coefficient of free FAD (11.3×10^3 M^{-1} cm^{-1}) at 465 nm [3]. Thionine acetate (Wako Pure Chemical Industries, with a purity of 90 % and a formal potential of −0.14 V vs. Ag|AgCl|KCl(sat.)), toluidine blue (Tokyo Chemical Industry, 80 %, −0.19 V), methylene blue hydrate (Tokyo Chemical Industry, 99.6 %, −0.17 V), and methylene green (Tokyo Chemical Industry, 88.3 %, −0.06 V) were used without further purification. All measurements were conducted at room temperature (25 ± 1 ^{0}C). Prior to each experiment, the solutions were purged with Ar for 10 min to remove O_2. The glucose stock solution was stored overnight to achieve mutarotative equilibrium.

2.2. Electrochemical behavior of polyphenothiazines in the presence or absence of FAD-GDH

Phenothiazines were electrochemically polymerized on the surface of a GC electrode by immersing the electrode into 1 mM monomer solutions in a 0.1 M phosphate buffer containing 0.1 M $NaNO_3$ and cycling the potential between −400 and 1200 mV vs. Ag|AgCl at a scan rate of 10 mV s^{-1} for ten cycles (Fig. S1) [12]. Cyclic voltammetry measurements using poly(phenothiazine)-modified GC electrodes as working electrodes were carried out in a 0.1 M phosphate buffer between −300 and 600 mV at a scan rate of 10 mV s^{-1} in the presence and absence of 0.1 M glucose and 1.0 μM FAD-GDH.

2.3. Glucose oxidation via constant-potential electrolysis

The polyMG-modified electrode was washed in magnetically stirred distilled water for 5 min to remove the adsorbed unreacted monomer (MG) or non-immobilized polyMG, and then 5 μL of FAD-GDH (45 μM in water) was dropped on the polymer layer. After the evaporation of water in a low-humidity cage (relative humidity < 3%, room temperature, 15 min), the electrode surface was covered with a dialysis membrane prepared from a cellulose-type dialysis tube (molecular weight cut-off = 12,000–14,000 Da, ASONE, Japan) and dipped into a pH 7, 0.1 M phosphate buffer solution. The effect of glucose concentration on the glucose oxidation current at 0.5 V was determined by successive additions of glucose to the electrolyte. The solution was gently stirred using a magnetic stirrer.

2.4. PolyMG modification of MgO-templated carbon and biofuel cells

MgO-templated carbon (MgOC) with an average pore size of 40 nm (Toyo Tanso, Japan) was drop-casted on the surface of the GC electrode as described in existing literature [20]. MG was polymerized on the MgOC electrode by cycling the potential between −400 and 900 mV at a scan rate of 10 mV s^{-1} for 10 cycles in a 0.1 M phosphate buffer containing 1 mM MG and 0.1 M $NaNO_3$. The BFC anode was fabricated as follows: The poly(vinylidene fluoride) solution (96 mg) and MgOC particles (48 mg) were mixed in 1 mL of *N*-methylpyrrolidone using a tip-type ultrasonicator (SMT UH-50, Tokyo, Japan). The carbon ink was dipped on TORAY carbon paper. The PolyMG was modified on MgOC-modified electrodes in a manner similar to the modification on the GC electrode. Finally, 10 μL cm^{-2} of FAD-GDH solution (25 mg mL^{-1}) was dropped onto the MgOC-modified electrode.

Biocathodes were fabricated according to methods described in the literature [22]. Polytetrafluoroethylene powder (50 mg, type 6 J, Du Pont-Mitsui Fluorochemicals, Japan) and MgOC powder (50 mg) in 3 mL of 2-propanol were mixed using a tip-type ultrasonicator. Carbon ink was applied to the 10 mm × 10 mm strips of carbon paper. After evaporation of the solvent, 60 μL cm^{-2} of bilirubin oxidase solution (50 mg mL^{-1} in distilled water) was dropped onto the MgOC-modified electrode. A BFC was constructed with a MgOC-modified anode and cathode with a cellophane membrane (Futamura Chemical, Japan). The performance of the BFC was analyzed using an electronic load device (PLZ164WA, Kikusui Electronic Corp., Japan).

2.5. Quartz crystal microbalance

Quartz crystal microbalance measurements were performed using QCA922A (Seiko EG&G Co., Ltd., Japan). 9-MHz AT-cut quartz crystal plates coated with carbon were used (Seiko EG&G), for which the projected surface area of the electrode was 0.196 cm^2. After the addition of FAD-GDH to the cell, the resonance frequency decreased with time and tended to reach the limiting value. The Sauerbrey equation [23] was used to convert the frequency change to the adsorbed mass of FAD-GDH.

3. Results and discussion

3.1. Mediated electron transfer via poly(phenothiazine) from FAD-GDH

Cyclic voltammetry was used to investigate the electrocatalytic activity of the poly(phenothiazine)-modified GC electrodes toward glucose oxidation in the presence of FAD-GDH (Fig. 1). The dashed curves in Fig. 1 show the cyclic voltammograms of poly(phenothiazine)-modified GC electrodes recorded in a pH 7 phosphate buffer in the absence of FAD-GDH, revealing the presence of two redox couples. The redox peaks with negative potential were ascribed to non-polymerized (adsorbed) monomers, while the peaks located at positive potentials were ascribed to poly(phenothiazine)s [12,16]. The midpoint potentials of poly(thionine) (polyTH), poly(Toluidine blue) (polyTB), poly(methylene blue) (polyMB), and poly(methylene green) (polyMG) were 0.00, 0.03, 0.05, and 0.12 V vs. Ag|AgCl|KCl(sta.), respectively. Although identical electropolymerization conditions (monomer concentration, electrolyte composition, potential range, sweep rate, and number of cycles) were used in all experiments, the amounts of surface-deposited polyphenothiazines varied. The generation of linkages between amine nitrogen and phenothiazine rings was identified as the most probable polymer formation mechanism [24], and the differences observed for polymer film formation were therefore attributed to the effects of steric hindrance or electron density differences. In particular, polyTH and polyMG showed lower redox peak heights than other polymers, which was explained by structural considerations. Although TH has two primary amine groups linked to the phenothiazine ring, other investigated monomers have one or two tertiary amine groups. Moreover, MG features a nitro group adjacent to a tertiary amine group, which may affect the structure of the proposed polyMG that features a branched skeleton based on ring-to-ring and nitrogen-to-ring couplings [12,15,16].

When 1.0 μM of FAD-GDH was added to the buffer solution (pH 7.0) containing 0.1 M glucose, a pronounced catalytic glucose oxidation current above 0.2 V was observed only for the polyMG-modified electrode (Fig. 1, solid curves). In the absence of glucose or in the absence of enzymes, no catalytic current of glucose oxidation was observed on polymer grafted electrodes, so it can be said that this increase in current was attributed to the electrochemical oxidation reaction of glucose via polyMG as redox mediator. All phenothiazine monomers acted as mediators of FAD-GDH [6], but polyMG showed the highest mediation activity toward FAD-GDH for catalytic current production. In addition, a catalytic wave was observed at the potential of polyMG and not at that of the monomer MG. This result also suggests the electron transfer scheme from the FAD to the electrode through the polyMG. The reactivity toward the monomer mediator was not affected by functional

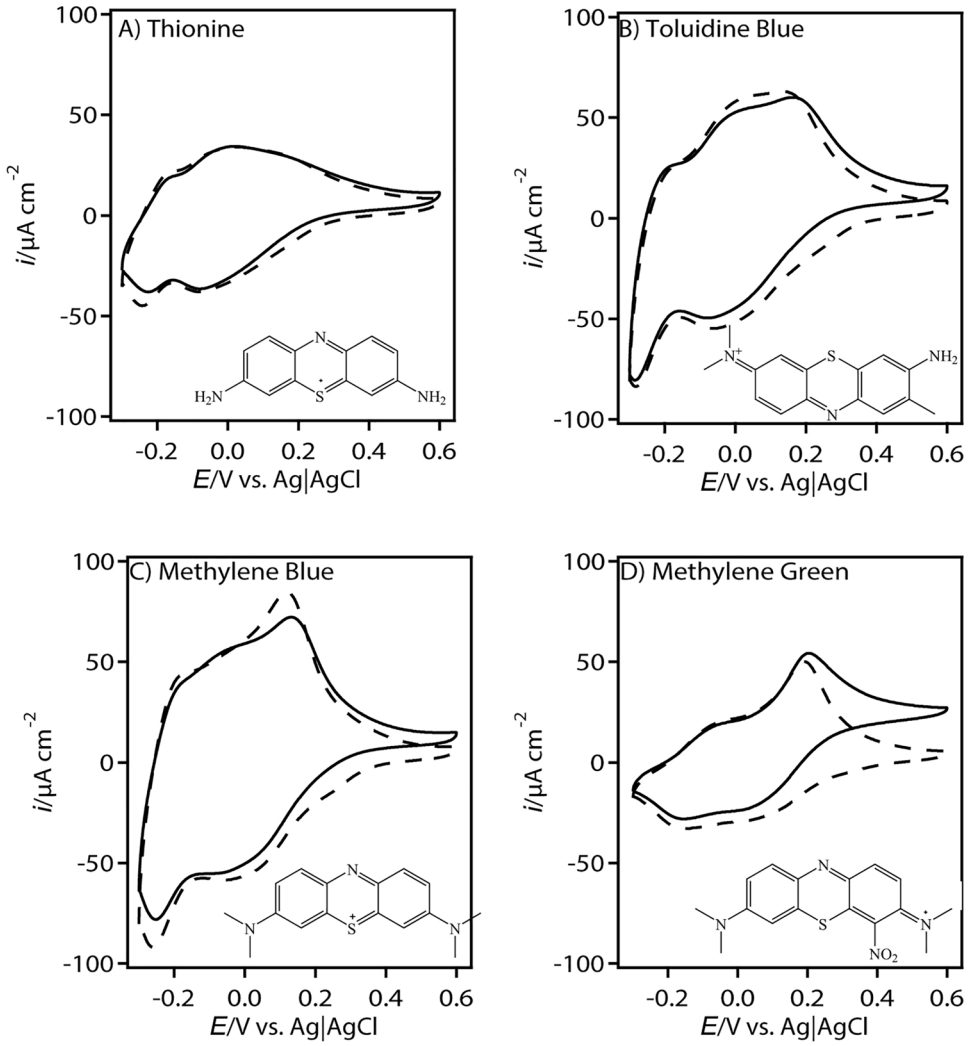

Fig. 1. Cyclic voltammograms of poly(phenothiazine)-modified GC electrodes in phosphate buffer (pH 7.0) in the absence (dashed curves) and presence of 1.0 μM FAD-GDH (solid curves)/0.1 M glucose. The scan rate was 10 mV s^{-1}.

groups such as side chains, which suggested that the structure of the polymer greatly influences—and/or the redox potential of the polymer influences—the reactivity with the enzyme. When GOx was used as an electrocatalyst instead of FAD-GDH, no glucose oxidation current was observed for the poly(phenothiazine)-modified electrode; polyMG access to the FAD site through the substrate binding pocket affected catalytic activity [6].

3.2. Effect of enzyme concentration and amount of polymer on the glucose oxidation current

The above catalytic current depended on the square root of the bulk concentration of FAD-GDH (panel A in Fig. 2, and Fig. S2 (A)), which indicated that the concentration polarization of oxidized and reduced forms of the redox mediator in the polymer layer resulted in the formation of a reaction layer in the vicinity of the electrode [25]. When the polyMG-modified electrode dipped in the FAD-GDH solution was shifted to a glucose solution without FAD-GDH, the electrode was not able to produce a glucose oxidation current. We also tested the adsorption of FAD-GDH on a poly(phenothiazine)-modified GC electrode using QCM measurements and observed that all exhibited the same adsorption capacity of 5 ± 1 pmol cm^{-2} for FAD-GDH adsorption. It can be concluded that FAD-GDH adsorption on poly(phenothiazine)s is not the only parameter that defines the possible electrical connection between FAD-GDH and the poly(phenothiazine)s and that the catalytic current was not affected by the amount of FAD-GDH adsorbed on the polymer surface.

To check if the electrocatalytic current depends on the amount of polyMG deposited on the GC electrode, we increased the number of potential scans during the electropolymerization of MG. We observed that the catalytic current density increased with an increasing number of electropolymerization cycles (panel B in Fig. 2). The peak potentials linearly increased with increasing numbers of electropolymerization cycles (Fig. S2 (B-1)), and the catalytic currents at 0.5 V depended on the square root of the cycle number (Fig. S2 (B-2)).

3.3. Dependence on glucose concentration

Fig. 3 shows the current changes during constant-potential electrolysis at 0.5 V in response to successive additions of glucose and reveals the dependence of the catalytic current on glucose concentration. The current increased linearly with increasing glucose concentration. The corresponding data were obtained in Ar and air atmospheres. This response was not affected by the presence of O_2 in the solution, which indicated that both FAD-GDH and polyMG were not sensitive to O_2. PolyMG has a high formal potential because the reduced form of polyMG is not sensitive to O_2 [26], while the reduced form of free MG with −0.06 V can be sensitive to dissolved oxygen. The reproducibility (coefficient of variation values) of the electrode response, as shown in Fig. 3, was less than 10 % using five different electrodes.

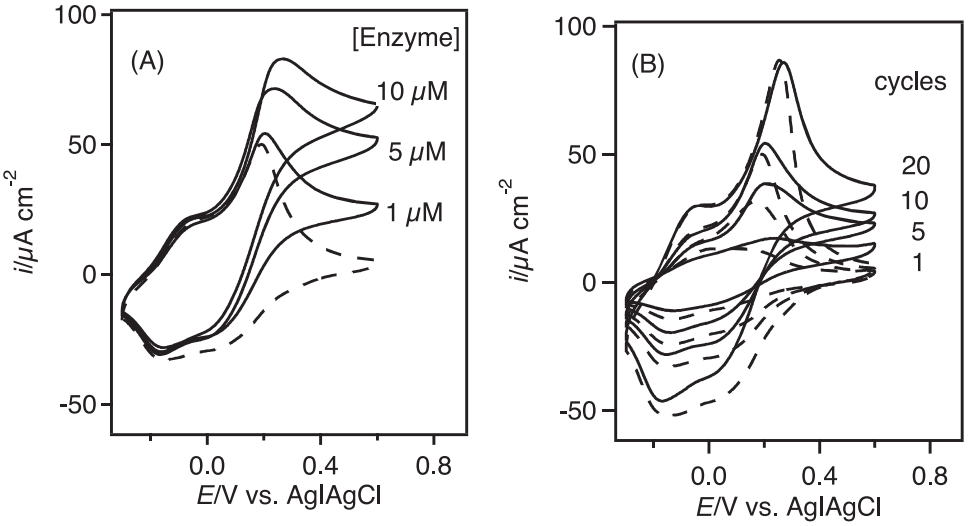

Fig. 2. Dependence of catalytic current for FAD-GDH-catalyzed glucose oxidation in phosphate buffer (pH 7.0) containing 0.1 M glucose on the concentration of FAD-GDH (A, ten cycles) and the number of polyMG deposition cycles (B, 1.0 μM FAD-GDH); in the absence (dashed line) and presence (solid line) of FAD-GDH. The scan rate was 10 mV s^{-1}.

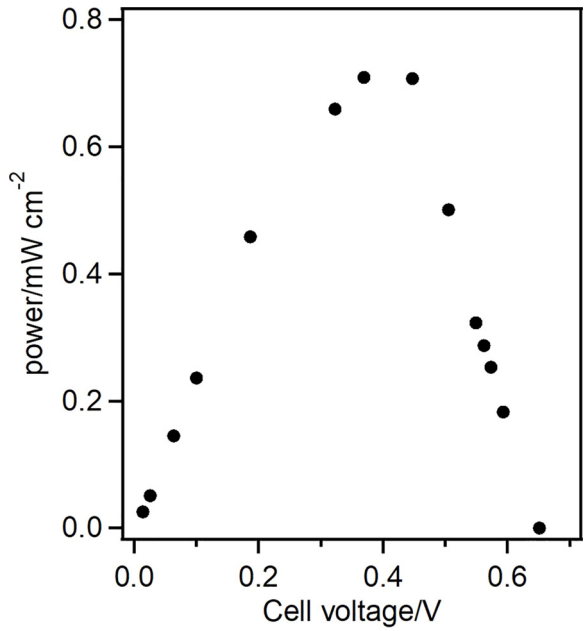

Fig. 3. Dependence of catalytic current density on glucose concentration. Closed and open circles represent data acquired in Ar and air atmospheres, respectively.

3.4. polyMG on a porous carbon electrode

The results presented in Fig. 2 also suggest an alternative strategy using porous carbon as an electrode material to efficiently increase the catalytic current density, since the electrochemical reaction is probably limited by the rate of electron diffusion within the polyMG layer. Low polyMG loadings (thin polyMG layer on non-porous electrode) led to a decreased rate of electron transfer between the enzyme and polyMG (as presented in Fig. 2B), which implies that the use of porous carbon coated with a thin layer of polyMG should improve both the rate of enzyme-polyMG encounters and that of electron diffusion through the polymer. It should be noted that without MG modification, no catalytic current was observed, which suggests that FAD-GDH has no direct electron transfer activity with the MgOC electrode. To form a thin polyMG film on the carbon surface, the potential scan range for the electropolymerization of polyMG was changed to a potential between −400 and 900 mV. Fig. 4(A) shows the cyclic voltammograms of the polyMG-modified MgOC electrode in the absence (dashed curve) and presence (solid curve) of 1.0 μM FAD-GDH in a pH 7 phosphate buffer containing 0.1 M glucose, revealing that the observed maximum current density by geometric surface area was almost 100 times higher than that observed for a non-porous GC electrode. Fig. 4(B) shows the dependence of the catalytic current density on the number of potential scans during the electropolymerization of MG, showing that the optimum number of scans was ten. Initially, the catalytic current increased with increasing polymer loading, but excessively large polymer quantities prevented the access of FAD-GDH to the polymer formed inside the carbon layer and hence decreased the usability of carbon.

The complete BFC was constructed using the polyMG-modified FAD-GDH bioanode and bilirubin oxidase biocathode with a cellophane membrane between the two bioelectrodes. The BFC was operated under air in a 0.1 M phosphate buffer (pH 7.0) containing 0.5 M glucose to examine cell performance. Fig. 5 shows the power density–voltage curve recorded in galvanostatic mode by monitoring the voltage response. The open circuit voltage of the EBFC was 0.65 V. The maximum current density was 2.5 mA cm^{-2}. The maximum power density delivered was 0.7 mW cm^{-2} at 0.45 V.

4. Conclusions

In summary, we demonstrated for the first time that electrochemically polymerized MG can mediate electron transfer between FAD-GDH and the electrode. The bulky polymer structure could be attributed to the functional groups of MG, thereby enabling effective electric contact between polyMG and the FAD site buried in the protein shell. Notably, polyMG and FAD-GDH showed O_2-insensitive current responses to glucose, and the corresponding glucose electrode was therefore proven suitable for use in enzymatic BFCs and glucose biosensors. Indeed, the thin polyMG layers formed on a porous carbon material with a high specific surface area result in enhanced performance by reducing the electron transfer distance. In this study, although the catalytic current on porous carbon reached 100 times higher than that on a non-porous carbon electrode, more improvement can be expected by optimizing the structure and arrangement of porous electrode materials.

CRediT authorship contribution statement

Nozomu Tsuruoka: Investigation, Methodology, Writing - original draft. **Silvia Sato Soto:** Investigation, Methodology. **Awatef Ben Tahar:** Supervision. **Abdelkader Zebda:** Supervision, Writing - review & editing. **Seiya Tsujimura:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - original

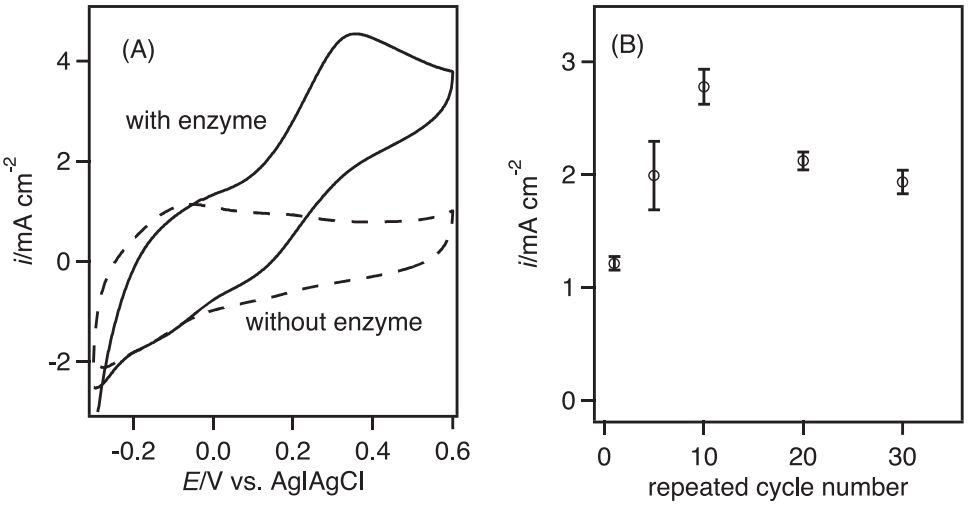

Fig. 4. (A) Cyclic voltammograms of the polyMG-modified MgOC electrode recorded in the absence (dashed curve) and presence (solid curve) of 1.0 μM FAD-GDH for cycle number = 10, (B) dependence of catalytic current density at 0.5 V on the number of MG modification cycles in the presence of 1.0 μM FAD-GDH. Phosphate buffer (pH 7) containing 0.1 M glucose was used as the electrolyte. The scan rate was 10 mV s^{-1}.

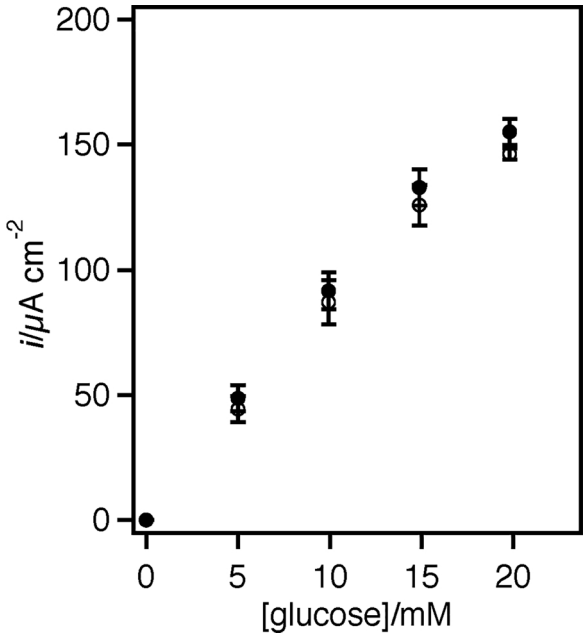

Fig. 5. Cell power curve as a function of cell voltage under air and room temperature (23 °C) conditions. 0.1 M phosphate buffer containing 0.5 M glucose.

draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2020.111065.

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